
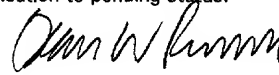


FORM PTO-1390 (REV. 1-98)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 41577/263898
<b>TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371</b>			U.S. APPLICATION NO. (if known, see 37 CFR 1.5) unknown <b>09/937068</b>
INTERNATIONAL APPLICATION NO. PCT/GB00/01104	INTERNATIONAL FILING DATE 23 March 2000 (23.03.00)	PRIORITY DATE CLAIMED 24 March 1999 (24.03.99)	
TITLE OF INVENTION VACCINE COMPOSITION			
APPLICANT(S) FOR DO/EO/US ALPAR, Hazire Oya; SOMAVARAPU, Satyanarayana; WILLIAMSON, Ethel Diane; BAILLIE, Leslie William James			
<p>Applicants herewith submit to the United States Designated/Elected Office (DO/EO/US) the following items and other information:</p> <ol style="list-style-type: none"> <li><input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.</li> <li><input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.</li> <li><input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 37 (b) and PCT Articles 22 and 39(1).</li> <li><input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</li> <li><input checked="" type="checkbox"/> A copy of the International Application as published (35 U.S.C. 371(c)(2))             <ol style="list-style-type: none"> <li><input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</li> <li><input type="checkbox"/> has been transmitted by the International Bureau.</li> <li><input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</li> </ol> </li> <li><input type="checkbox"/> A translation of the published International Application into English (35 U.S.C. 371(c)(2)).</li> <li><input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))             <ol style="list-style-type: none"> <li><input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</li> <li><input type="checkbox"/> have been transmitted by the International Bureau.</li> <li><input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</li> <li><input checked="" type="checkbox"/> have not been made and will not be made.</li> </ol> </li> <li><input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).</li> <li><input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</li> <li><input type="checkbox"/> A translation of the annexes of the International Preliminary Examination Report under PCT Article 36</li> <li><input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.197 and 1.98</li> <li><input checked="" type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</li> <li><input checked="" type="checkbox"/> A FIRST preliminary amendment.             <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</li> <li><input type="checkbox"/> A substitute specification.</li> <li><input type="checkbox"/> A change of power of attorney and/or address letter.</li> <li><input checked="" type="checkbox"/> Other items or information:             <ol style="list-style-type: none"> <li>Certification Under 37 CFR 1.10</li> </ol> <p>I hereby certify that this document is being mailed to Box PCT, Commissioner for Patents, Washington, D.C. 20231, via "Express Mail Post Office to Addressee" on this 20th day of September, 2001, Express Mail Label No. EL209599012US</p> <p><u>Angela M. Rossi</u> Angela M. Rossi</p> <ol style="list-style-type: none"> <li>Version with markings to show changes made</li> <li>International Preliminary Examination Report with six amended sheets</li> </ol> </li> </ol>			

U.S. APPLICATION NO. (if known, see 37 CFR 1.41) unknown		INTERNATIONAL APPLICATION NO. PCT/GB00/01104		ATTORNEY'S DOCKET NUMBER 41577263898	
17. <input checked="" type="checkbox"/> The following fees are submitted BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):				CALCULATIONS PTO USE ONLY	
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO				\$1,000.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO				\$860.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO				\$710.00	
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)				\$690.00	
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)				\$100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$860.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$0.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$	
Total claims	25	05	X \$18.00	\$90.00	
Independent claims	05	02	X \$80.00	\$160.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$270.00	\$0.00	
TOTAL OF ABOVE CALCULATIONS =				\$1,110.00	
Reduction of 1/2 for filing by small entity, if applicable.				\$0.00	
SUBTOTAL =				\$1,110.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$0.00	
TOTAL NATIONAL FEE =				\$1,110.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40 per property				\$40.00	
TOTAL FEES ENCLOSED =				\$1,150.00	
				Amount to be refunded:	\$
				charged:	\$
<p>a. <input checked="" type="checkbox"/> Check in the amount of \$1,150.00 is enclosed.</p> <p>b. <input type="checkbox"/> Please charge my Deposit Account No. 11-0855 in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.</p> <p>c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 11-0855.</p>					
<p>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</p>					
Customer No. 23370					
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John S. Pratt, Esq.		SIGNATURE			
KILPATRICK STOCKTON LLP		PATENT TRADEMARK OFFICE			
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## IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

Applicants: Hazire Oya ALPAR, Satyanarayana  
SOMAVARAPU, Ethel Diane WILLIAMSON,  
And Leslie William James BAILLIE

International  
Application No.: PCT/GB00/01104

U.S. Serial No.:

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Filing Date: 23 March 2000 (23.03.00)

U.S. Filing Date: 20 September 2001 (20.09.01)

For: VACCINE COMPOSITION

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Commissioner for Patents  
Washington, D.C. 20231

Attorney Docket No. 41577/263898  
Date: 20 September 2001

**PRELIMINARY AMENDMENT**

Sir:

Kindly amend the above-identified patent application prior to examination:

In the Claims

3. (Amended) A composition according to claim 1 wherein the said adjuvant chemical can act as an immunostimulant.

4. (Amended) A composition according to claim 1 wherein the said adjuvant chemical is selected from one or more of;

- A) poly-ornithine, for example of molecular weight from 5 to 150kDa;
- B) water soluble vitamins or vitamin derivatives such as vitamin E TPGS (d-alpha tocophenyl polyethylene glycol 1000 succinate),

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"Vaccine Composition"  
Filed: 20 September 2001  
PRELIMINARY AMENDMENT

- C) cationic pluronics which are block copolymers or surfactants which are positively charged, in particular with  $\text{NH}_2^+$  groups
- D) complexing agents which form complexes with fatty acids such as deoxycholic acid, or
- E) cyclodextrins and their derivatives such as dimethyl  $\beta$  cyclodextrin.
5. (Amended) A composition according to claim 1 wherein the carrier comprises a particle.
10. (Amended) A composition according to claim 7 wherein the microsphere comprises poly-(L-lactide).
11. (Amended) A composition according to claim 1 wherein the ratio of the chemical (ii) to the carrier is from 99:1 to 9:1 w/w.
12. (Amended) A composition according to claim 1 which is adapted for administration to a mucosal surface or is suitable for parenteral administration.
15. (Amended) A method of protecting a mammal against infection, which method comprises administration of a composition according to claim 1 to a mammal.

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Filed: 20 September 2001  
PRELIMINARY AMENDMENT

20. (Amended) A microsphere according to claim 18 which further comprises an agent that is capable of generating a protective immune response in an animal to which it is administered.

22. (Amended) A pharmaceutical composition comprising a microsphere according to claim 19.

Respectfully submitted,



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Marked up version of claims showing changes made

3. (Amended) A composition according to claim 1 [or claim 2] wherein the said adjuvant chemical can act as an immunostimulant.

4. (Amended) A composition according to [any one of the preceding] claim[s] 1 wherein the said adjuvant chemical is selected from one or more of;

- A) poly-ornithine, for example of molecular weight from 5 to 150kDa;
- B) water soluble vitamins or vitamin derivatives such as vitamin E TPGS (d-alpha tocophenyl polyethylene glycol 1000 succinate),
- C) cationic pluronics which are block copolymers or surfactants which are positively charged, in particular with  $\text{NH}_2^+$  groups
- D) complexing agents which form complexes with fatty acids such as deoxycholic acid, or
- E) cyclodextrins and their derivatives such as dimethyl  $\beta$  cyclodextrin.

5. (Amended) A composition according to [any one of the preceding] claim[s] 1 wherein the carrier comprises a particle.

10. (Amended) A composition according to [any one of] claim[s] 7 [to 9] wherein the microsphere comprises poly-(L-lactide).

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Filed: 20 September 2001  
PRELIMINARY AMENDMENT

11. (Amended) A composition according to [any one of the preceding] claim[s]  
1 wherein the ratio of the chemical (ii) to the carrier is from 99:1 to 9:1 w/w.

12. (Amended) A composition according to [any one of the preceding] claim[s]  
1 which is adapted for administration to a mucosal surface or is suitable for parenteral  
administration.

15. (Amended) A method of protecting a mammal against infection, which  
method comprises administration of a composition according to [any one of] claim[s] 1 [to  
13] to a mammal.

20. (Amended) A microsphere according to claim 18 [or claim 19] which  
further comprises an agent that is capable of generating a protective immune response in an  
animal to which it is administered.

22. (Amended) A pharmaceutical composition comprising a microsphere  
according to [any one of] claim[s] 19 [to 22].

Vaccine Composition

The present invention relates to compositions which are particularly useful for delivering medicaments, and in particular vaccines. Compositions are suitable for parenteral administration or non-parenteral administration. This includes compositions for administration to mucosal surfaces, for example intranasal formulations, or for topical administration to the skin. The invention further comprises methods of treating individuals using the composition, methods of preparing the composition and components for use in the compositions.

A prime objective in the field of vaccination is the development of a non-parenteral immunisation regimen which facilitate induction of comparable levels of systemic immunity to that elicited by conventional sub-cutaneous and intra-muscular injections.

The nasopharyngeal passages and pulmonary regions of the respiratory tract represent potential targets for the systemic delivery of peptidergic drugs and vaccines. The relative ease with which therapeutic agents can be inhaled, or introduced into the nose, make these modes of immunisation attractive in terms of probable patient compliance. Furthermore, respiratory mucosae offer certain morphological, physiological and immunological advantages over other non-parenteral sites in terms of immunisation, particularly against pathogenic entities which affect or utilise mucosal surfaces as portals of entry. This is because effective vaccination against these pathogens normally requires mucosae to be adequately protected with locally produced antibodies of the secretory IgA (sIgA) isotype. Whilst mucosal surfaces are usually poorly protected with IgA following parenteral administration of vaccines, it is now apparent that successful delivery of antigenic material to immunoresponsive elements in mucosa-associated lymphoid tissue (MALT) can result in vigorous stimulation of the mucosal arm of



the immune system. By means of the common mucosal immune system (CMIS) it is feasible that several anatomically disparate mucosal surfaces could be protected through mucosal administration of a vaccine at a single site. Mucosal vaccination offers the added advantage that some degree of systemic immunity can be induced in concert with local responses due to translocation of antigenic material from sub-epithelial compartments to systemic immunoresponsive tissues.

Despite the logistical and immunological factors which favour non-parenteral immunisation, simple mucosal application of antigenic proteins, for example in the gastrointestinal or respiratory tracts, is usually ineffectual in terms of vaccination. Enzymatic or chemical destruction, combined with poor absorption into sub-epithelial compartments dictate that mucosally administered vaccines usually require some form of adjuvant or delivery vehicle. One approach is to encapsulate antigenic material within microparticulate polymeric carriers, such as poly-DL-lactide (PLA) microspheres (Vaccine 1994, 12, 5-11). Such procedures serve to protect labile vaccines from luminal degradation and enhance absorption into mucosal and systemic compartments (J.H. Eldridge et al., Seminars in Hematology, (1993), 30, 16-25). There is good evidence that microencapsulation may also adjuvantise by converting soluble antigenic molecules into particulate species, thus promoting vaccine uptake into antigen presenting cells (APC) (Y. Tabata et al., Adv. Polym. Sci. (1990), 94, 107-141, L. Vidard et al., J. Immunol. (1996), 156, 2809-2818, N. Van Rooijen, Immunol. Today (1990) 11, 436-439) or microfold cells (M-cells) in lymphoid follicles (R.I. Walker et al., Vaccine, 12, 387, 1994, D.T. O'Hagan et al., Vaccine, 1989, 7, 421-424, P.G. Jenkins et al., J. Drug Targeting, 1995, 3, 79-81). Nasal delivery of microsphere formulation of vaccine has also been reported (A.J. Almeida et al., J. Pharm & Pharmacology, 25, 198-203 1993, H.O. Alpar et al., J. Drug Targeting 2/2, 147-149, 1994, A.J. Almeida et al., J. Drug Targeting 3(b), 255-467 1996).

Although until recently comparatively under-investigated, the intra-nasal (i.n.) route is an attractive one for the mucosal delivery of vaccinal entities. The nasal epithelium is  
5 accessible and is less exclusive to high molecular weight molecules.

The thickness of the mucus blanket covering respiratory epithelium is relatively thin compared to that of other mucosae,  
10 for example the gut where it is in the region of 500 times thicker. Substantially reduced concentrations of proteolytic enzymes and extremes of pH exist in the respiratory tract compared with the gastrointestinal tract.

15 Furthermore, it is now delineated that nasal associated lymphoids tissues (NALT) have a lymphoepithelium which, like that in the intestinal mucosa, contain M-cells for selective antigen uptake (P. Brandenburg, Immunology of the Lung and Upper Respiratory Tract, (ed. Bienenstock J.) McGraw-Hill, New York,  
20 1984, 28-95). Hence NALT plays an analogous role to other MALT, such as the gut associated lymphoid tissues (GALT), in terms of antigen surveillance and induction of mucosal and systemic immunological responses.

25 The applicants have found that a particular range of chemicals, when included in formulations, can increase or enhance the effect of biologically active agents and in particular vaccines. A particular effect noted appears to be an immunostimulant or adjuvant effect. They are noted particularly when the  
30 compositions are administered to mucosal surfaces, but are also observed when compositions are applied parenterally, for example intramuscularly (i.m. administration). Topical formulations for administration to skin surfaces may also benefit from the inclusion of these chemicals.

As used herein, the term "immunostimulant" refers to an adjuvant which stimulates the immune system of a host animal to which it is administered and thereby increases the protective effect produced by a protective antigen administered to that animal, as compared to the effect which would be produced by administration of the protective antigen alone.

According to the present invention there is provided a pharmaceutical composition comprising

- 10 (i) a biologically active agent;
- (ii) an adjuvant chemical which increases the effect of the biologically active agent, said chemical selected from one or more of:
  - 15 A) a polyamino acid,
  - B) a vitamin or vitamin derivative,
  - C) cationic pluronics,
  - D) a clathrate,
  - E) a complexing agent,
  - F) cetrinides;
  - 20 G) an S-layer protein; or
  - H) Methyl-glucamine; and
- (iii) a pharmaceutically acceptable carrier or diluent; provided that when the chemical (ii) above is selected from D) or E), the biologically active agent (i) is an agent which is capable of generating a protective immune response in an animal to which it is administered.

Particular examples of suitable chemicals are those of groups, A, B, C, D, E, F and H defined above. A further particular group of chemicals are those of group G above.

As used herein, the expression "cationic pluronics" includes both pluronics which include cations, as well as those which have been treated such that they are bound to cationic moieties (cationised pluronics).

Suitable biological agents (i) include drugs and therapeutic molecules such as vaccines, antivirals, antibiotics, antifungals, antiparasitics as well as oligonucleotides used in therapies and vaccines.

5

However in a preferred embodiment, the biologically active agent is an agent that is capable of generating an immune response in an animal to which it is administered and most preferably a protective immune response. Thus the compositions are suitably used as vaccines including those which rely on oligonucleotides or other nucleic acid sequences. In this case, the immunostimulant properties of the compounds A-H are used.

Suitably the said adjuvant chemical is soluble in water.

15 Suitably the composition is suitable for non-parenteral administration for example to mucosal surfaces or for topical application to the skin. Particularly preferred compositions are suitable for administration to mucosal surfaces.

20 Alternatively, the composition is suitable for parenteral administration for example by intramuscular (i.m.) administration.

Administration to mucosal surfaces may be effected by oral application, by pulmonary application, for example by intra-tracheal administration, or particularly by intra-nasal application. In particular, the compositions of the invention are administered by the intra-nasal route.

30 Examples of adjuvant chemicals in category (A) above include polyamino acids such poly-ornithine, for example of molecular weight from 5 to 150kDa.

Particular examples of adjuvant chemicals in category (B) above are vitamins or vitamin derivatives such as vitamin E or

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derivatives for example vitamin E TPGS (d-alpha tocopheryl polyethylene glycol 1000 succinate).

5 Particular cationic pluronics in category (C) above, are block copolymers or surfactants which are positively charged, in particular with  $\text{NH}_2^+$  groups. These are available commercially for example from ICI Ltd (UK) sold under the trade names P101 and P121. These may be used alone, but may preferably be used in combination with other adjuvants.

10

Examples of clathrates in category (D) above include in particular cyclodextrins and their derivatives such as dimethyl  $\beta$  cyclodextrin.

15 Suitable complexing agents in category (E) above are bile salts, in particular those which form complexes with fatty acids such as deoxycholic acid.

20 Examples of cetrimides in category (F) are quaternary ammonium compounds used as preservatives.

Such chemicals may further enhance the mucosally produced effect of the biologically active agent by acting as absorption enhancers and/or bioadhesive compounds and/or solubilisers.

25

Preferably the chemicals include at least one of the group selected from (A), (B), (C) or (D). When the chemical comprises a vitamin or derivative of (B) above, it is suitably present in concentrations in excess of 0.2%w/v, preferably in excess of 2%w/v.

30

Carriers or diluents used as (iii) above may vary depending upon the particular nature of the biologically active agent (i) and the further chemical (ii). They may comprise pharmaceutically acceptable solvents such as water in which the biologically active agent (i) and the further chemical (ii) are dissolved.

35

This type of formulation is particularly suitable when (i) is also water-soluble.

Compositions in the form of solutions of this type suitably contain from 0.1 to 30% w/v and preferably from 1 to 20% w/v of component (ii) above, depending upon the solubility of component (ii).

For many applications however, it has been found preferable that components (i) and (ii) are microencapsulated in a polymeric material and thus the carrier (iii) is a particulate carrier such as a microparticle, nanocapsule or liposome.

Thus in a particular embodiment, the invention provides a pharmaceutical composition, which composition comprises particles comprising

(i) biologically active agent;  
(ii) an adjuvant chemical which increase the biological effect of the composition, said chemical being soluble in water and being selected from one or more of:

- A) a polyamino acid; or
- B) a vitamin or vitamin derivative; and
- C) cationic pluronic
- D) a clathrate,
- E) a complexing agent,

(iii) a material capable of forming a particle.

Particular examples will include a compound of (A), (B), (D) or (E). Cationic pluronics of (C) may be included either within the particles, or as a component of the dosing mixture or both.

Compositions of this type, are, as mentioned above, particularly suitable for administration to a mucosal surface, although they may be of a suitable size to allow parenteral administration.

Particularly suitable particles are liposomes and microspheres. Liposome forming chemicals for use as (iii) above are well known in the art and include lipids with a hydrophilic end region and a hydrophobic region and the opposite end of the molecule.

- 5    Microspheres or microparticles (sometimes called microcapsules) will generally be prepared using polymeric materials as is known in the art.

- 10    Suitably, the adjuvant chemical which increases the biological effect of the composition in this case is a polymeric material which is different to the polymeric material, where present, of item (iii) above.

- 15    The polymeric material (iii) above used in the compositions of the invention may comprise one or more polymers, for example having molecular weights of from 2kDa or more. In particular, the polymeric material (iii) is a high molecular weight polymer, for example of molecular weight in excess of 94kDa, for example of 100kDa or more.

- 20    The use of high molecular weight polymers in the encapsulation of a tetanus vaccine for intramuscular administration has been described (Vaccine 1994, 12, 4, 299-306). A formulation of microencapsulated ricin toxoid vaccine which is applied  
25    intranasally has also been described (Vaccine 1994, 14, 11 1031). However, in that case, high molecular weight polymer microparticles (94kDa) were less effective than those prepared from a copolymer of lower molecular weight (72kDa).

- 30    A particularly suitable polymeric material for use in the compositions of the invention comprises poly-(L-lactide) or PLA but other polymeric materials such as poly(lactic/glycolic acid) PLGA, polycyanoacrylates, polyanhydrides or polycaprolactones as are known in the art may be employed.

Suitably the component (ii) is present in the composition in an amount of from 0.1% to 10%w/w.

The compositions of the invention may optionally further  
5 comprise agents which stabilise emulsions such as  
polyvinylalcohol or methyl cellulose.

Other conventional reagents may be added. These include other  
known composition components such as colouring agents and  
10 preservatives and in particular cetrimide. These are suitably  
present in amounts of from 0.1 to 0.7%w/v.

In a particular embodiment, the microspheres or liposomes used  
in the compositions may comprise S-layer proteins. These may be  
15 present as the sole immunostimulant compound, or they may be  
used in combination with other immunostimulants such as those  
defined in A)-F) and (H) above. The S-layer proteins may be  
present in the microspheres or liposomes themselves. In a  
preferred embodiment however, they are provided as a coating on  
20 the surface of the microspheres or liposomes. Particularly  
preferred S-layer proteins are derived from a bacterium against  
which the biologically active agent produces a protective immune  
response. It has been shown (Sleyr et al., Crystalline  
bacterial cell surface proteins. Biotechnology Intelligence  
25 Unit, 1996, R.G. Landes Company and Academic Press Inc.) that  
the stability of liposomes can be increased by such coatings.  
S-layer proteins are found on the surface of most bacteria and  
form a regular two dimensional array known as an S-layer.  
Isolated S-layer proteins are able to form entropy driven  
30 monomolecular arrays in suspension, and on the surface of  
structures such as liposomes.

S-layer proteins have not heretofore, been applied to  
microspheres however. Consequently, microspheres containing  
35 such proteins, in particular in the form of a coating on the  
surface of the microsphere, form a further aspect of the



invention. These microspheres are preferably utilised as vaccines in that they carry one or more immunogenic agents which produce a protective immune response when administered to an animal. These agents may be within or distributed throughout the  
5 microsphere and may additionally be complexed to the S-layer protein coating.

Preferably, the S-layer protein is derived from the bacterial pathogen against which protection is sought. For example,  
10 microspheres employed as in vaccines against *B.anthraxis* may be coated with *B. anthracis* S-layer protein.

Compositions of the invention are particularly suitable for mucosal, especially intranasal application. They may comprise  
15 simple solutions of the components as described above, or microparticles per se which are optionally preserved, for example by lyophilisation, or the microparticles may themselves be combined with a pharmaceutically acceptable carrier or excipient. Examples of suitable carriers include solid or  
20 liquid carriers as is understood in the art.

Microparticles used in the compositions of the invention will suitably be of an average size of from 0.1 $\mu$ m to 10 $\mu$ m in diameter.

25 These compositions may be used to deliver a range of biologically active agents including drugs and pharmaceutical chemicals as well as hormones such as insulin.

30 These compositions have been found to be particularly effective in the administration of biologically active agent which is capable of generating a protective immune response in an animal, particularly a mammal, to which it is administered. Examples of such agents include antigenic polypeptides as well as nucleic  
35 acid sequences which may encode these polypeptides and which are known as "DNA" vaccines. Both the level and the longevity of

the immune response is increased when these formulations are employed.

As used herein the expression "polypeptide" encompasses proteins  
5 or epitopic fragments thereof.

Suitable polypeptides are sub-unit vaccines and others, such as diphtheria toxoid, tetanus toxoid and *Bacillus anthracis* protective antigen (PA).

10

In a preferred embodiment, the composition of the invention comprises a biologically active agent which is capable of generating a protective immune response against *Yersinia pestis*. The agent is suitably a sub-unit vaccine, for example as  
15 described in WO 96/28551. The vaccine described and claimed there comprises a combination of the V antigen of *Y. pestis* or an immunologically active fragment thereof or a variant of these, and the F1 antigen of *Y. pestis* or an immunologically active fragment thereof or a variant of these.

20

As used herein, the term "fragment" refers to a portion of the basic sequence which includes at least one antigenic determinant. These may be deletion mutants. One or more epitopic region of the sequence may be joined together.

25

The expression "variant" refers to sequences of nucleic acids which differ from the base sequence from which they are derived in that one or more amino acids within the sequence are substituted for other amino acids. Amino acid substitutions may  
30 be regarded as "conservative" where an amino acid is replaced with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino acids of a different type. Broadly speaking, fewer non-conservative substitutions will be possible  
35 without altering the biological activity of the polypeptide. Suitably variants will be at least 60% homologous, preferably at

least 75% homologous, and more preferably at least 90% homologous to the base sequence. Homology in this instance can be judged for example using the algorithm of Lipman-Pearson, with Ktuple:2, gap penalty:4, Gap Length Penalty:12, standard  
5 PAM scoring matrix (Lipman, D.J. and Pearson, W.R., Rapid and Sensitive Protein Similarity Searches, *Science*, 1985, vol. 227, 1435-1441).

Preferably, vaccine compositions will further comprise a further  
10 known adjuvant in order to enhance the immune response to the biologically active material administered. Suitable adjuvants include pharmaceutically acceptable adjuvants such as Freund's incomplete adjuvant, alhydrogel, aluminium compounds and, preferably adjuvants which are known to up-regulate mucosal  
15 responses such as CTB, the non-toxic pentameric B subunit of cholera toxin (CT), or mutant heat-labile toxin (mLT) of *E.coli*.

A further potential immunostimulant compound which may be included in the compositions are polycationic carbohydrates such  
20 as those described and claimed in copending International Patent application of the applicants of even date, derived from British Patent Application Nos. 9906694.6 and 9906696.1. Particular examples of polycationic carbohydrate which act as immunostimulants include a chitin derivative, cationic  
25 polypeptide, cationic polyamino acid, a quaternary ammonium compound or a mixture thereof. Especially preferred are chitin derivatives such as chitosan, or water-soluble chitin derivatives such as alkylated chitosans or salts thereof. Particular examples are trimethylchitosans such as those  
30 described by A.F. Kotze et al. *Pharm Res.* (1997) 14: 1197-1202.

A particular aspect of the invention comprises a method of producing a pharmaceutical composition, which method comprises encapsulating a biologically active agent in a particle  
35 comprising a first material which is capable of forming a particle, in the presence of an adjuvant chemical which

increases the effect of the biologically active agent when administered to a mucosal surface, said chemical being soluble in water and being selected from one or more of:

- 5 A) a polyamino acid,
- B) a vitamin or vitamin derivative,
- C) cationic pluronics,
- D) a clathrate,
- E) a complexing agent,
- F) cetrimides; or
- 10 H) Methyl-glucamine.

In particular the said chemical is selected from one or more of

- A) a polyamino acid;
- B) a vitamin or vitamin derivative,
- 15 D) a clathrate,
- E) a complexing agent.

In an alternative group, the said chemical is selected from one or more of

- 20 A) a polyamino acid,
- B) a vitamin or vitamin derivative,
- C) cationic pluronics,
- D) a clathrate,

- 25 Preferred examples of particle forming materials and adjuvant (effect enhancing) chemicals are as set out above.

- Methods of forming liposomes are well known in the art. They include dispersion of dehydrated phospholipid films into an aqueous medium, emulsion techniques and lyophilisation methods as are well known in the art.
- 30

- The adjuvant chemical may be incorporated within the microcapsule, or at the surface, of preferably is distributed throughout the microcapsule including the surface.
- 35

- Microencapsulated compositions of the invention are suitably prepared using a double emulsion solvent evaporation method. Briefly, the biologically active agent, suitably in a lyophilised state, is suspended in an aqueous solution of the polymer such as polyvinyl alcohol (PVA) and the adjuvant chemical. A solution of further polymer, in particular high molecular weight polymer in an organic solvent such as dichloromethane, is added with vigorous mixing. The resultant emulsion is then dropped into a secondary aqueous phase, also containing polymer (PVA or the like) and optionally also the adjuvant material with vigorous stirring. After addition, the organic solvent is allowed to evaporate off and the resultant microspheres separated.
- 15 The compositions of the invention will suitably comprise an appropriate dosage unit of the active agent. This will vary depending upon the nature of the active agent being employed, the nature of the patient, the condition being treated and other clinical factors. In general however, the composition of the invention will comprise approximately 2 to 10 wt% of active ingredient.

In microcapsule formulations, the amount of high molecular weight first polymer in the composition will be of the order of 70 to 99wt% of the composition, and suitably from 90 to 99wt% of the polymer components will be the first polymer.

The amount of adjuvant chemical present in the compositions will be sufficient to produce the required effect. This will vary depending upon the nature of the chemical but will generally be of the order of 0.1 to 10 wt % of the composition.

In use, a reasonable dosage for nasal administration would be from about 0.05g to 0.2g.

Preferred compositions of the inventions are vaccine compositions. Thus, in a further aspect, the invention provides a method of protecting a mammal against infection, which method comprises administration of a vaccine composition as described above in particular to a mucosal surface, such as a nasal surface, of a mammal.

The applicants have demonstrated that it is possible to protect experimental animals from inhalation challenge with various pathogens including diphtheria, tetanus and *Y. pestis* through i.n. administration of a combined sub-unit vaccine. The adjuvantisation of these sub-units is advantageous in increasing the immune response as is microencapsulation of the sub-units in accordance with the invention. The high molecular weight polymer utilised in the compositions of the invention appears to be particularly well suited to intra-nasal delivery.

In a further aspect, the invention provides the use of an adjuvant chemical as defined above as an immunostimulant in the production of a vaccine for use in prophylactic or therapeutic treatment.

The invention will now be particularly described by way of example with reference to the accompanying drawings in which:

Figures 3 illustrates the specific serum antibody responses following a single nasal application of 1µg V and 5µg F1 antigens of *Yersinia pestis* in compositions according to the invention:

Figures 1, 2 and 4 illustrates the immune response to nasally delivered tetanus toxoid (TT) using compositions according to the invention where ES is glycodeoxycholic acid, CYC is dimethyl  $\beta$  cyclodextrin, and VET is Vitamin E TPGS and PO is polyornithine;

Climatic conditions		Soil conditions		Plant growth		Yield		Quality	
Parameter	Unit	Parameter	Unit	Parameter	Unit	Parameter	Unit	Parameter	Unit
Temperature	°C	Moisture	%	Height	cm	Grain yield	kg/ha	Protein content	%
Light intensity	μmol/m²/s	pH		Stem diameter	cm	Straw yield	kg/ha	Starch content	%
Relative humidity	%	EC	dS/m	Root length	cm	Seed yield	kg/ha	Cellulose content	%
CO <sub>2</sub> concentration	ppm	Organic matter	%	Chlorophyll content	SPAD	Harvest index		Lignin content	%
Soil temperature	°C	Nitrogen	mg/kg	Stomatal conductance	mol/m²/s	Waterlogging tolerance		Phenolic compounds	mg/g
Air temperature	°C	Phosphorus	mg/kg	Transpiration rate	mmol/m²/s	Root to shoot ratio		Flavonoids	mg/g
Soil temperature	°C	Potassium	mg/kg	Photosynthesis rate	μmol/m²/s	Seedling vigor		Antioxidant activity	μg/g
Relative humidity	%	Sulfur	mg/kg	Chlorophyll fluorescence	Fv/Fm	Seedling height	cm	Protein solubility	%
CO <sub>2</sub> concentration	ppm	Zinc	mg/kg	Stomatal conductance	mol/m²/s	Seedling biomass	g	Protein digestibility	%
Soil temperature	°C	Copper	mg/kg	Transpiration rate	mmol/m²/s	Seedling root length	cm	Protein stability	%
Air temperature	°C	Manganese	mg/kg	Photosynthesis rate	μmol/m²/s	Seedling stem diameter	cm	Protein functionality	%
Soil temperature	°C	Iron	mg/kg	Chlorophyll fluorescence	Fv/Fm	Seedling root diameter	cm	Protein shelf life	days
Relative humidity	%	Selenium	mg/kg	Stomatal conductance	mol/m²/s	Seedling root volume	cm³	Protein storage	g/g
CO <sub>2</sub> concentration	ppm	Chlorine	mg/kg	Transpiration rate	mmol/m²/s	Seedling root weight	g	Protein utilization	g/g
Soil temperature	°C	Boron	mg/kg	Photosynthesis rate	μmol/m²/s	Seedling root length	cm	Protein quality	g/g
Air temperature	°C	Magnesium	mg/kg	Chlorophyll fluorescence	Fv/Fm	Seedling root diameter	cm	Protein safety	g/g
Soil temperature	°C	Calcium	mg/kg	Stomatal conductance	mol/m²/s	Seedling root volume	cm³	Protein value	g/g
Relative humidity	%	Sodium	mg/kg	Transpiration rate	mmol/m²/s	Seedling root weight	g	Protein use	g/g
CO <sub>2</sub> concentration	ppm	Aluminum	mg/kg	Photosynthesis rate	μmol/m²/s	Seedling root length	cm	Protein health	g/g
Soil temperature	°C	Strontium	mg/kg	Chlorophyll fluorescence	Fv/Fm	Seedling root diameter	cm	Protein life	g/g
Air temperature	°C	Barium	mg/kg	Stomatal conductance	mol/m²/s	Seedling root volume	cm³	Protein energy	g/g
Soil temperature	°C	Lithium	mg/kg	Transpiration rate	mmol/m²/s	Seedling root weight	g	Protein power	g/g
Relative humidity	%	Vanadium	mg/kg	Photosynthesis rate	μmol/m²/s	Seedling root length	cm	Protein strength	g/g
CO <sub>2</sub> concentration	ppm	Chromium	mg/kg	Chlorophyll fluorescence	Fv/Fm	Seedling root diameter	cm	Protein speed	g/g
Soil temperature	°C	Molybdenum	mg/kg	Stomatal conductance	mol/m²/s	Seedling root volume	cm³	Protein size	g/g
Air temperature	°C	Antimony	mg/kg	Transpiration rate	mmol/m²/s	Seedling root weight	g	Protein shape	g/g
Soil temperature	°C	Bismuth	mg/kg	Photosynthesis rate	μmol/m²/s	Seedling root length	cm	Protein form	g/g
Relative humidity	%	Thallium	mg/kg	Chlorophyll fluorescence	Fv/Fm	Seedling root diameter	cm	Protein color	g/g
CO <sub>2</sub> concentration	ppm	Lead	mg/kg	Stomatal conductance	mol/m²/s	Seedling root volume	cm³	Protein taste	g/g
Soil temperature	°C	Mercury	mg/kg	Transpiration rate	mmol/m²/s	Seedling root weight	g	Protein smell	g/g
Air temperature	°C	Cadmium	mg/kg	Photosynthesis rate	μmol/m²/s	Seedling root length	cm	Protein touch	g/g
Soil temperature	°C	Barium	mg/kg	Chlorophyll fluorescence	Fv/Fm	Seedling root diameter	cm	Protein sound	g/g
Relative humidity	%	Strontium	mg/kg	Stomatal conductance	mol/m²/s	Seedling root volume	cm³	Protein weight	g/g
CO <sub>2</sub> concentration	ppm	Lithium	mg/kg	Transpiration rate	mmol/m²/s	Seedling root weight	g	Protein height	g/g
Soil temperature	°C	Vanadium	mg/kg	Photosynthesis rate	μmol/m²/s	Seedling root length	cm	Protein width	g/g
Air temperature	°C	Chromium	mg/kg	Chlorophyll fluorescence	Fv/Fm	Seedling root diameter	cm	Protein depth	g/g
Soil temperature	°C	Molybdenum	mg/kg	Stomatal conductance	mol/m²/s	Seedling root volume	cm³	Protein surface	g/g
Relative humidity	%	Antimony	mg/kg	Transpiration rate	mmol/m²/s	Seedling root weight	g	Protein area	g/g
CO <sub>2</sub> concentration	ppm	Bismuth	mg/kg	Photosynthesis rate	μmol/m²/s	Seedling root length	cm	Protein volume	g/g
Soil temperature	°C	Thallium	mg/kg	Chlorophyll fluorescence	Fv/Fm	Seedling root diameter	cm	Protein mass	g/g
Air temperature	°C	Lead	mg/kg	Stomatal conductance	mol/m²/s	Seedling root volume	cm³	Protein density	g/g
Soil temperature	°C	Mercury	mg/kg	Transpiration rate	mmol/m²/s	Seedling root weight	g	Protein length	g/g

Figures 5-6 illustrate the immune response to nasally delivered Diphtheria toxoid (DT) using compositions according to the invention where BS is glycodeoxycholic acid, CYC is dimethyl  $\beta$  cyclodextrin, and VET is Vitamin E TPGS;

5

Figure 7 shows the results of photon correlation spectroscopy (PCS) on particles produced for use in the invention, showing that the number mean diameter ( $d_n$ ) and the volume mean diameter ( $d_v$ ) were both around 150nm; and

10

Figure 8 illustrates the immune response to Diphtheria toxoid (DT) in various formulations applied by i.m. routes, where P101 is a pluronic 101 and P121 is a block co-polymer available from ICI Ltd, UK.

15

#### Example 1

##### The effect of adjuvant chemicals.

20

Twelve groups of five (n=5) BALB/c mice were intranasally immunised with admixed F1 (5  $\mu$ g) and V (1  $\mu$ g). The five treatment groups received the subunits in conjunction with either:

- 1 Antigens free in 138kDa polyornithine (0.2-1%w/v conc.)
- 2 Microspheres including 138kDa polyornithine (0.2-1%w/v conc.)
- 3 Free in 30-70KDa polyornithine (0.2%w/v)
- 4 Free in 5-15kDa polyornithine (0.2%w/v)
- 5 Free in  $\beta$ -cyclodextrins (2.5%w/v)
- 6 Microspheres in  $\beta$ -cyclodextrins (2.5%w/v)
- 7 Free in deoxycholic acid (0.25%w/v)
- 8 Free in Vitamin E TPGS (2.5%w/w)
- 9 Free in Vitamin E TPGS (0.2% w/v)
- 10 Free in chitosan HCL (0.2%w/v)
- 11 Free in phosphate buffered saline (PBS)
- 12 Microspheres in PBS

A further group of animals acted as a control.

Mice were lightly anaesthetised with an inhaled gaseous mixture of 3% (v/v) halothane (RMB Animal Health Ltd., UK) in oxygen (300cm<sup>3</sup> min<sup>-1</sup>) and nitrous oxide (100cm<sup>3</sup> min<sup>-1</sup>) for i.n. dosing procedures. Each mouse received a 15µl volume of liquid administered with a micropipette. Tail vein blood samples were taken on day 14, and serum was analysed for the presence of anti-V and anti-F1 IgG antibodies using an indirect ELISA protocol (Eyles, J. E. et al. Vaccine (1998) 16:698-707).

The results are shown in Figure 1. This clearly shows that other compounds, in particular, poly-L-ornithine either free or in microspheres, β-cyclodextrins, deoxycholic acid and Vitamin E TPGS (the latter being present in amounts of 2.5% w/v) produced enhanced results.

This study has identified that absorption enhancers, with potential applications for increasing the bioavailability of non-parenterally administered peptidergic drugs, can also act to improve humoral immunity to mucosally applied subunit vaccines.

#### Example 2

##### Immune responses to nasally delivered tetanus toxoid (TT)

Further tests were carried out using the methodology of Example 1 but replacing the *Yersinia pestis* antigens with tetanus toxoid. Mice were dosed on day 1 with 5 LF toxoid and on day 49 with 2.5 LF toxoid. The toxoids were in solution in combination with a variety of adjuvant chemicals in various concentrations. The results are shown in Figures 2-4.

With these enhancers or immunostimulants, titres for primary responses were improved approximately 100 times and secondary responses between 1000 to 20000 times compared to free antigen.

#### Example 3

##### Immune responses to nasally delivered diphtheria toxoid (DT)



Example 2 was repeated on selected members of the enhancers using diphtheria toxoid in place of tetanus toxoid. The results are shown in Figures 5 and 6. Again, similar levels of enhancement are noted.

5

#### Example 4

#### Immune responses to intramuscularly delivered diphtheria toxoid (DT)

Eight formulations of pluronic/chitosan nanoparticles were prepared, by a simple sonication method. Deacylated high molecular weight chitosan was obtained from Fluka. Three process variables were : type of Pluronic used (P101, P121 obtained from ICI Limited, UK), volume of pluronic added (75µl or 200µl per 2ml water), and addition or omission of chitosan. Briefly, to 2ml of double-distilled water, a small volume (75µl or 200µl) of the appropriate pluronic liquid (P101 or P121) was added. Mixtures were vortexed for one minute and sonicated for a further one minute. For coating of the pluronic particles, 100µl of a solution of 0.1% w/v high molecular weight chitosan in 2% w/v glacial acetic acid was added to 100µl of each formulation. Finally, the diphtheria toxoid (DT) was adsorbed to the 200µl of coated and non-coated particles by the addition of 12µl of a solution of DT in water (4450lf units per ml). This preparation is a colloidal dispersion in water with a mean particle diameter of generally between 100-600nm. An example of a typical photon correlation spectroscopy (PCS) printout is shown in Figure 7.

Following characterisation of the prepared particulate formulations, groups of four or five female Balb/c mice were given a single dose of 50µl intramuscularly. The total equivocal dose for each animal was 5Lf units of DT. The final concentration of pluronics in the dosing medium was 5% (v/v) and chitosan 0.05% (w/v). Animals were bled periodically and ELISAs

carried out to determine serum levels of anti-DT specific IgG titres.

Mean serum anti-DT IgG titres are shown herewith in Figure 8.

- 5 Surprisingly it was found that chitosan-pluronic composition produced much higher immune response compared to pluronics or with free antigen alone.

#### Example 5

- 10 S-Layer protein extraction and purification from *Bacillus anthracis* strains RBA91 and SM91 and microspheres containing them

- Bacillus anthracis* produces two S-layer (surface layer) proteins: EA1 (Extractable Antigen) and Sap (Surface Array Protein) which form ordered paracrystalline arrays exterior to  
15 the cell-wall (Etienne-Toumelin et al. 1995: Journal of Bacteriology, 177(3): 614-620; Farchaus et al. 1995, Journal of Bacteriology 177:2481-2489 and Mesnage et al. 1997, Molecular Microbiology, 23(6):1147-1155). The proteins may be isolated  
20 using the method of Etienne-Toumelin et al. 1995 supra.

- In this method SPY medium (Etienne Toumelin et al. 1995 supra.) agar plates, supplemented with spectinomycin, are inoculated with the *B. anthracis* mutant strains RBA91 and SM91 (Mesnage et  
25 al, 1997 supra.). The plates are incubated overnight at 37°C.

- Late the next day, some of the biomass from each plate is re-suspended in sterile saline to an OD<sub>540</sub> of approximately 1.3 in each case. Prewarmed SPY broth (500ml in 1000ml flasks),  
30 supplemented with spectinomycin, was prepared fresh for each of the two mutants, saline suspension (400µl) was used to inoculate each flask. The cultures were then incubated with shaking at 37°C, at approximately 200rpm for 16-18 hours.

- 35 OD<sub>540</sub> readings were then taken from each culture and those with results of 1.5 or more selected. The culture broths were

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- decanted into sterile Sorvall/Beckmann centrifuge pots and centrifuged at 10000rpm for 30 minutes at 4°C. The supernatant was discarded and the pellets re-suspended in 5M guanidine hydrochloride at pH 8.0 (25ml) in sterile 50ml Falcon tubes.
- 5 The re-suspended pellet mixtures were incubated for 2 hours at room temperature in a shaking incubator at approx. 150 rpm.

- The extracts were then decanted into sterile 40ml Sorvall/Beckmann centrifuge tubes and centrifuges at 6000 rpm,
- 10 for 10 minutes at 4°C. The supernatants were decanted into sterile 30ml Universals. A set of 15ml Slide-a-Lyzer™ dialysis cassettes (Pierce, USA) are prepared and 15ml volumes of the supernatants are loaded by syringe needle into each cassette. Each cassette is placed in 1litre of 26mM Tris-HCl at pH 5.0 in
- 15 a glass beaker. The samples were dialysed overnight at 4°C in a refrigerator.

- The S-layer proteins obtained in this way were then adsorbed onto microspheres which contained *B. anthracis* protective
- 20 antigen (PA) as the biologically active agent.

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## Claims

1. A pharmaceutical composition comprising

(i) a biologically active agent;

5 (ii) an adjuvant chemical which increases the effect of the biologically active agent, said chemical selected from one or more of:

A) a polyamino acid,

B) a water soluble vitamin or vitamin derivative,

10 C) positively charged cationic pluronics,

D) a clathrate,

E) a complexing agent,

F) cetrimides;

G) an S-layer protein

15 H) Methyl-glucamine; and

(iii) a pharmaceutically acceptable carrier or diluent, subject to the following provisos

a) when the chemical (ii) above is selected from D) or E), the biologically active agent is an agent which is capable of  
20 generating a protective immune response in an animal to which it is administered;

b) when the chemical (ii) above is selected from A) and the biologically active agent is an agent which is capable of generating a protective immune response in an animal to which it  
25 is administered, the composition is for administration to a mucosal surface,

c) when the chemical (ii) above is selected from C) and the biologically active agent is an agent which is capable of generating a protective immune response in an animal to which it  
30 is administered, the composition does not contain a polyacrylic acid, and

d) where the chemical (ii) above is selected from G) and the biologically active agent is an agent which is capable of generating a protective immune response in an animal to which it  
35 is administered, the carrier or diluent of (iii) is a microsphere or liposome.

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2. A composition according to claim 1 wherein biologically active agent is an agent that is capable of generating a protective immune response in an animal to which it is administered.

5

3. A composition according to claim 1 or claim 2 wherein the said adjuvant chemical can act as an immunostimulant.

4. A composition according to any one of the preceding claims wherein the said adjuvant chemical is selected from one or more of;

- 10 A) poly-ornithine, for example of molecular weight from 5 to 150kDa;  
B) water soluble vitamins or vitamin derivatives such as vitamin E TPGS (d-alpha tocophenyl polyethylene glycol 1000 succinate),  
15 C) cationic pluronics which are block copolymers or surfactants which are positively charged, in particular with  $\text{NH}_2^+$  groups  
D) complexing agents which form complexes with fatty acids such as deoxycholic acid, or  
20 E) cyclodextrins and their derivatives such as dimethyl  $\beta$  cyclodextrin.

5. A composition according to any one of the preceding claims wherein the carrier comprises a particle.  
25

6. A composition according to claim 5 wherein the particle is a microsphere or liposome.

7. A composition according to claim 6 which comprises a  
30 microsphere.

8. A composition according to claim 7 wherein the microsphere is prepared using a high molecular weight polymer.

35 9. A composition according to claim 8 wherein the polymer has a molecular weight of 100kDa or more.

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10. A composition according to any one of claims 7 to 9 wherein the microsphere comprises poly-(L-lactide).
11. A composition according to any one of the preceding claims wherein the ratio of the chemical (ii) to the carrier is from 99:1 to 9:1 w/w.
12. A composition according to any one of the preceding claims which is adapted for administration to a mucosal surface or is suitable for parenteral administration.
13. A composition according to claim 2 which further comprises a further adjuvant.
14. A method of producing a prophylactic or therapeutic vaccine, which method comprises encapsulating a polypeptide which is capable of producing a protective immune response in a first polymeric material which has a high molecular weight, in the presence of a second polymeric material which increases the biological effect of the composition.
15. A method of protecting a mammal against infection, which method comprises administration of a composition according to any one of claims 1 to 13 to a mammal.
16. A method according to claim 15 wherein the composition is applied to a mucosal surface.
17. A method according to claim 16 wherein the mucosal surface comprises an intranasal surface.
18. A microsphere comprising a polymeric carrier and an S-layer protein.
19. A microsphere according to claim 18 wherein said S-layer protein is coated on the surface of the microsphere.

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20. A microsphere according to claim 18 or claim 19 which further comprises an agent that is capable of generating a protective immune response in an animal to which it is administered.

5

21. A microsphere according to claim 20 wherein one or more of said agents are linked to the S-layer protein.

22. A pharmaceutical composition comprising a microsphere according to any one of claims 19 to 22.

10

23. A pharmaceutical composition according to claim 22 wherein said composition is a vaccine, intended to produce a protective immune response against a bacterium, and said S-layer protein is derived from said bacterium.

15

24. The use of a chemical selected from

20

- A) a polyamino acid,
- B) a water soluble vitamin or vitamin derivative,
- C) positively charged cationic pluronics,
- D) a clathrate,
- E) a complexing agent,
- F) cetrinides;
- G) an S-layer protein; or
- H) Methyl-glucamine

25

as an immunostimulant, provided that in the case of A), the immunostimulant is applied to a mucosal surface, in the case of C, the compound is used in the absence of a polyacrylic acid.

30 25. The use of an adjuvant chemical selected from

35

- A) a polyamino acid,
- B) a water soluble vitamin or vitamin derivative,
- C) positively charged cationic pluronics,
- D) a clathrate,
- E) a complexing agent,
- F) cetrinides;
- G) an S-layer protein; or
- H) Methyl-glucamine

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as an immunostimulant in the production of a vaccine for use in prophylactic or therapeutic treatment, provided that in the case of A), the immunostimulant is used in a vaccine which is applied to a mucosal surface, and in the case of C), the compound is used  
5 in the absence of a polyacrylic acid.

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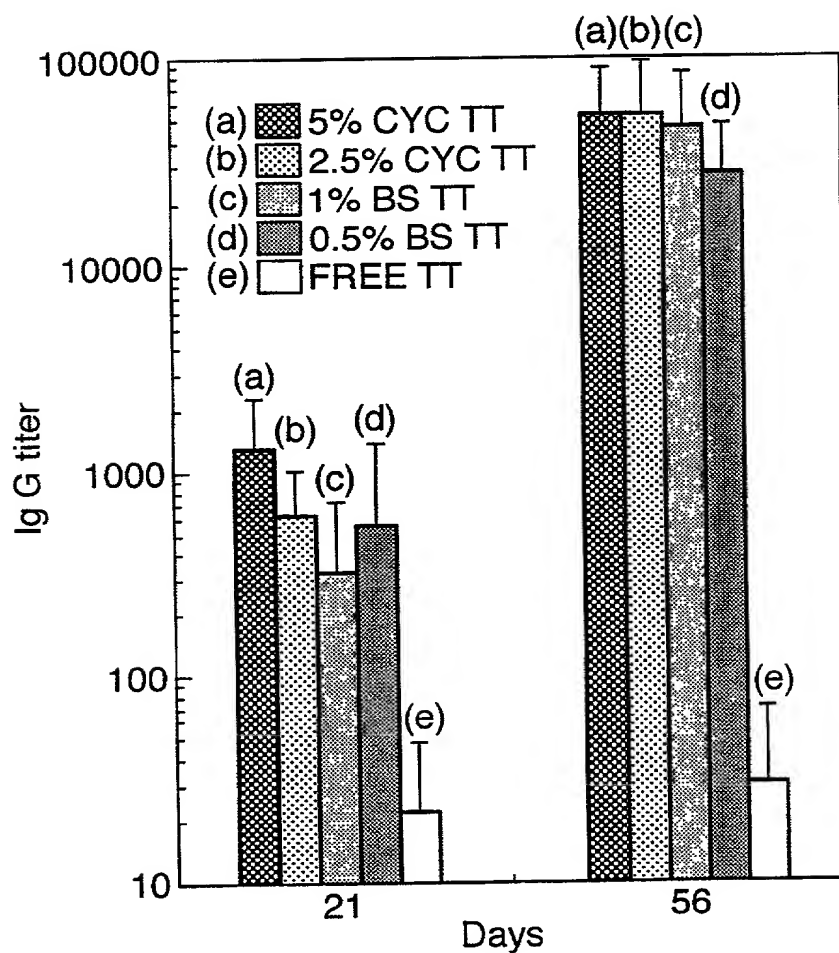
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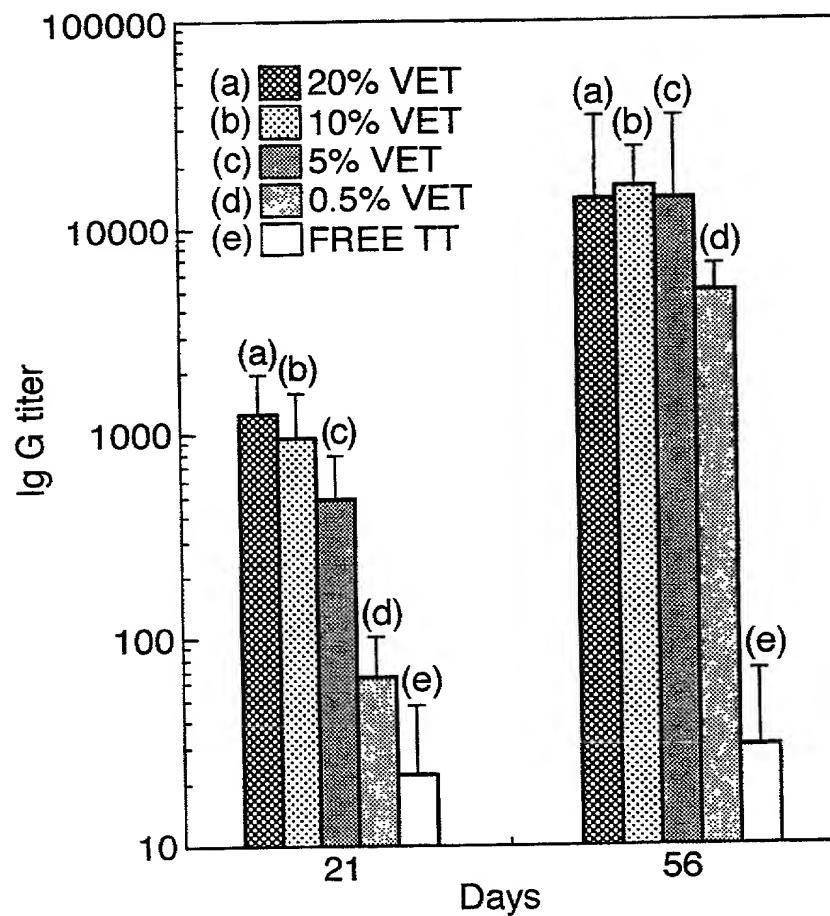
Fig.1.



Mice dosed on day 1 with 5 LF toxoid and on day 49 with 2.5 LF toxoid

BS = Glyco deoxy cholic acid  
CYC = Dimethyl (cyclodextrin)

Fig.2.



Mice dosed on day 1 with 5 LF toxoid and on  
day 49 with 2.5 LF toxoid

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Fig.3.

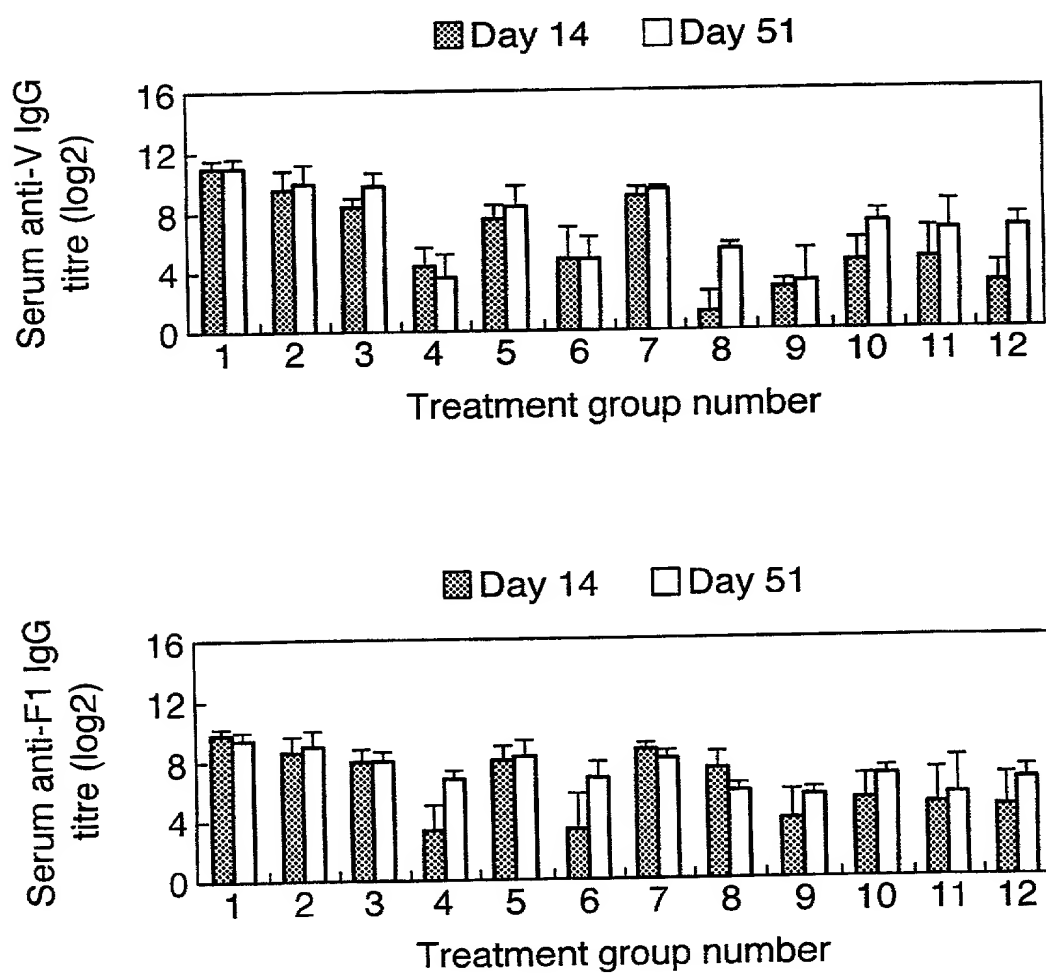
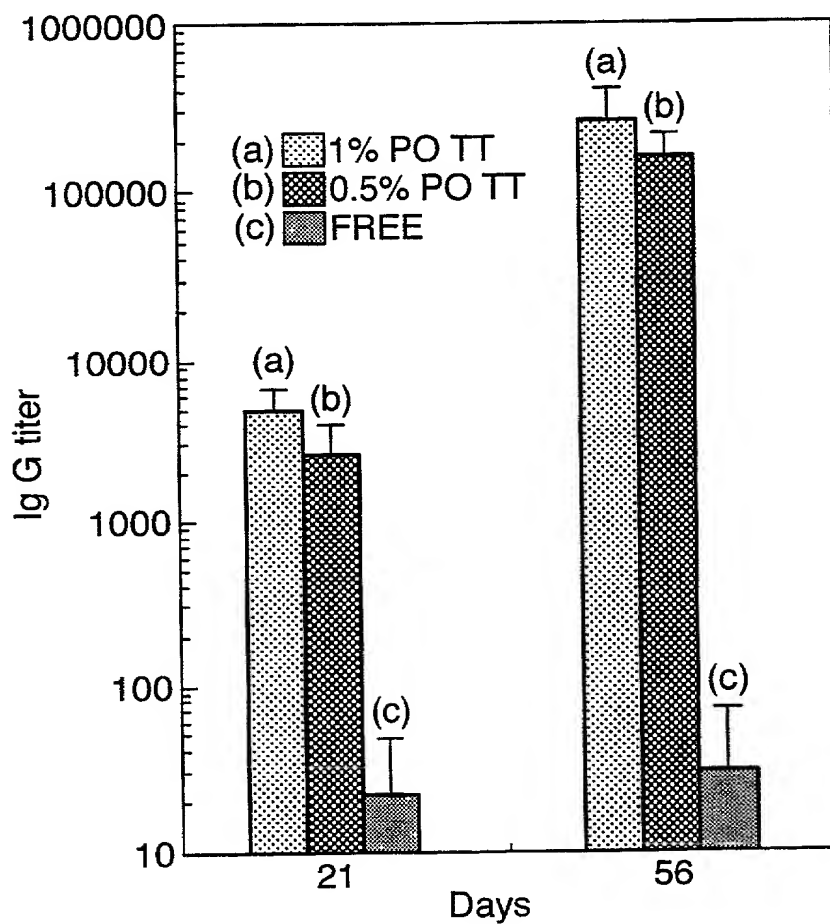


Fig.4.

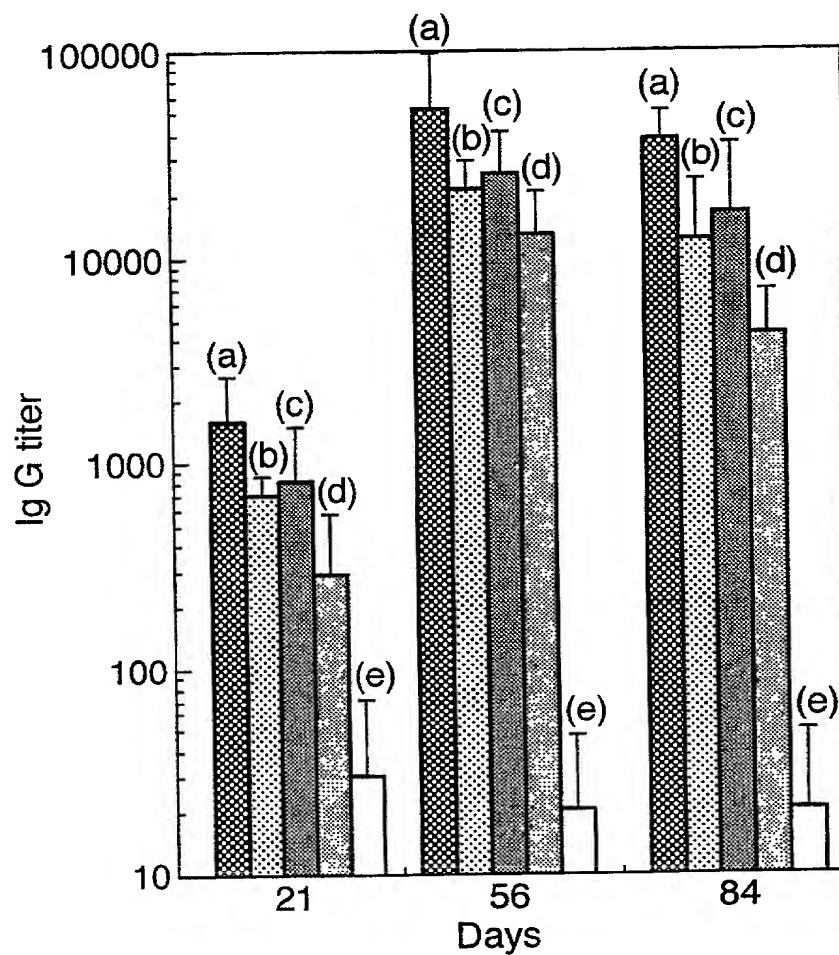


Mice dosed on day 1 with 5 LF toxoid and on day 49 with 2.5 LF toxoid

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Fig.5.

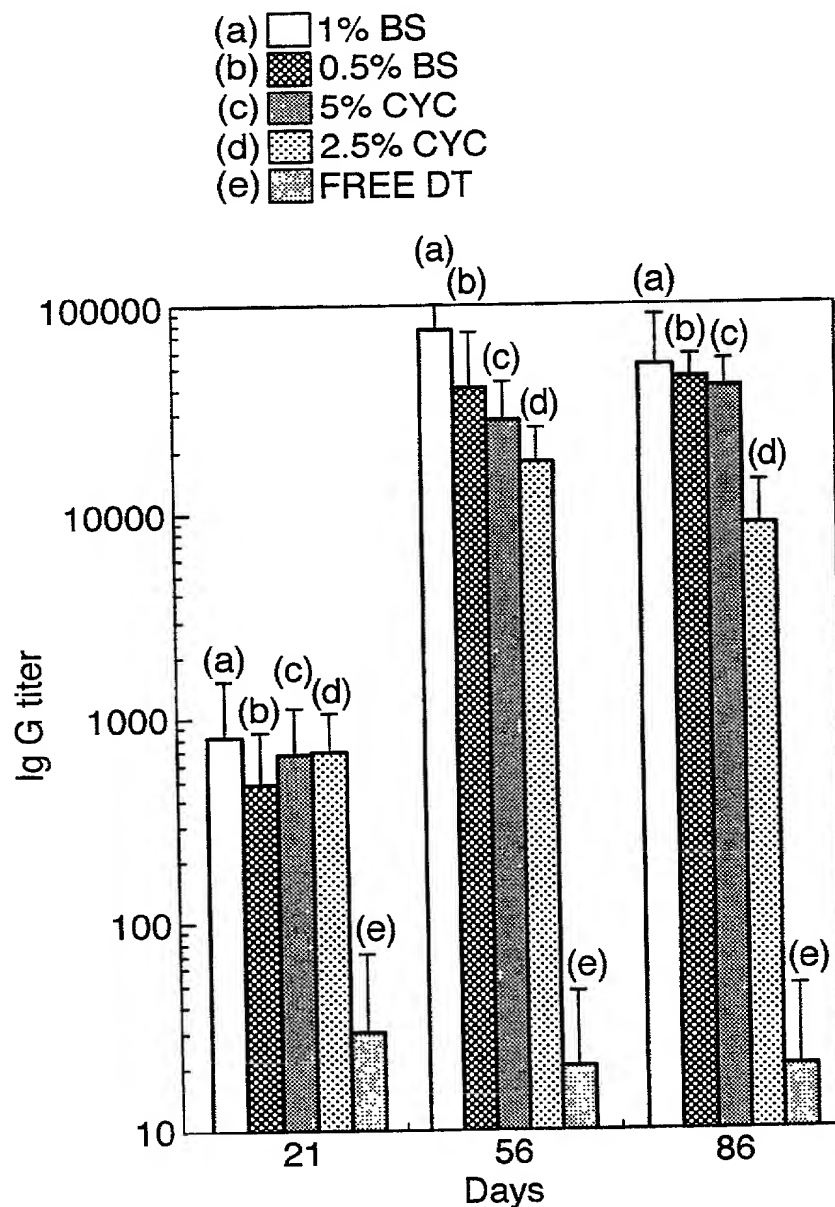
- (a) 20% VET  
(b) 10% VET  
(c) 5% VET  
(d) 0.5% VET  
(e) FREE DT



Mice dosed on day 1 with 5 LF toxoid and on  
day 49 with 2.5 LF toxoid

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Fig.6.



Mice dosed on day 1 with 5 LF toxoid and on  
day 49 with 2.5 LF toxoid

BS = Glyco deoxy cholic acid  
CYC = Dimethyl (cyclodextrin)

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Fig.7.

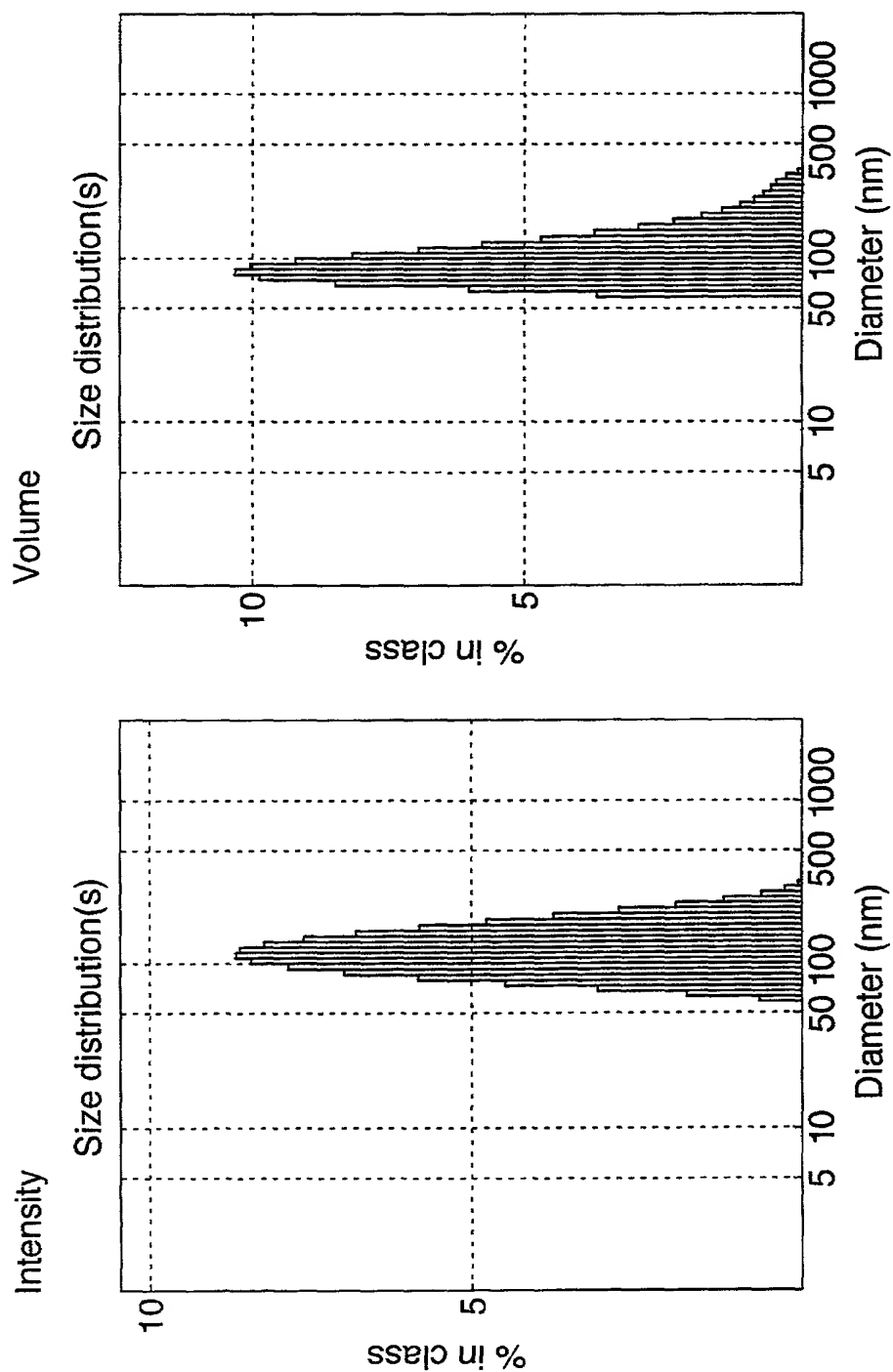
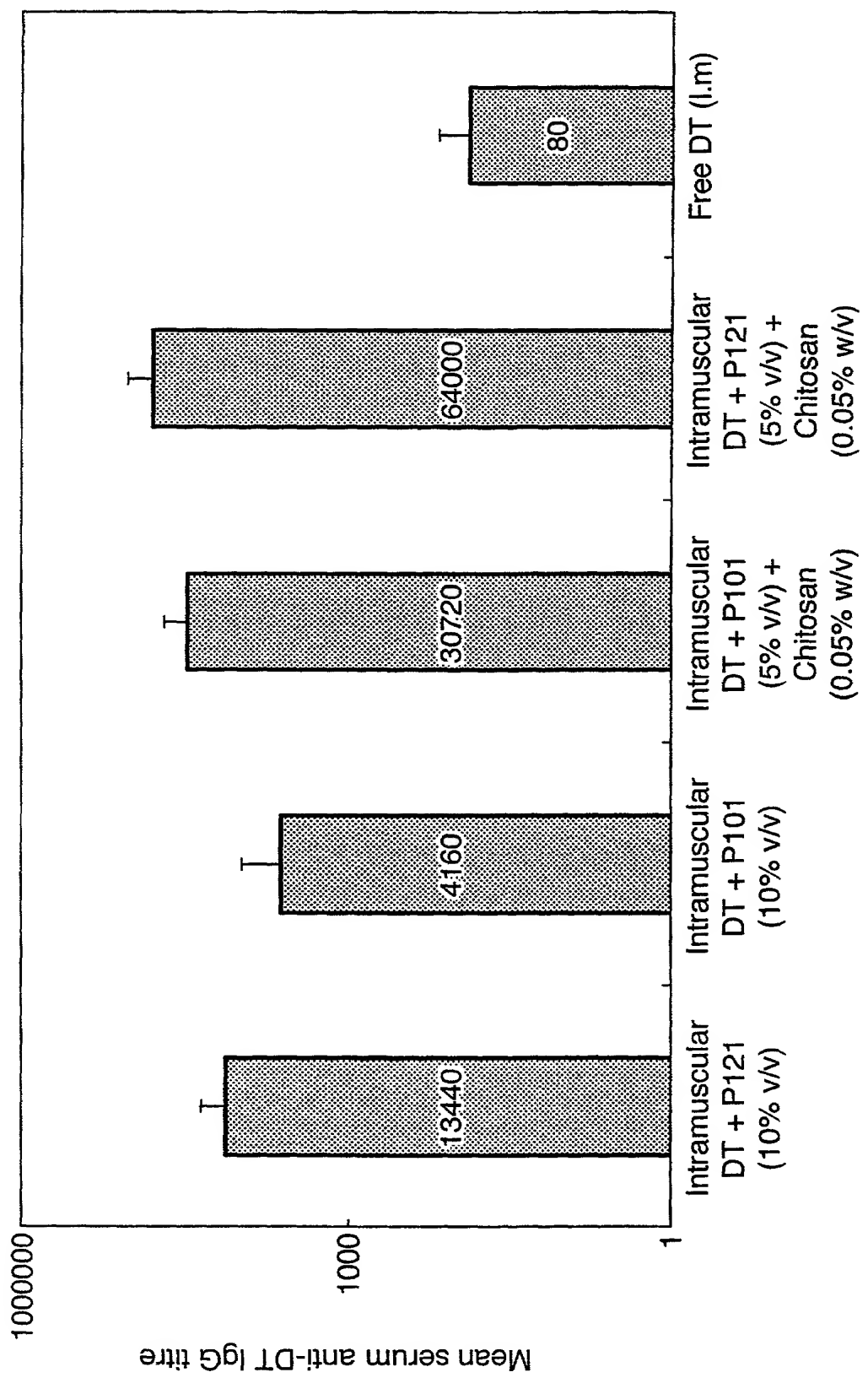


Fig.8.





**DECLARATION FOR PATENT APPLICATION**☒ Original☐ Supplemental☐ Substitute☐ PCT

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below), or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**VACCINE COMPOSITION****(Title of the Invention)**

the specification of which (check one)

☐ is attached hereto☐ was filed on \_\_\_\_\_ as U. S. Application Serial Number \_\_\_\_\_

☒ was filed as PCT International Application Number PCT/GB00/01104 on 23 March 2000  
and was amended under PCT Article 19 on 17 April 2001

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 (a) - (d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified, by checking the box below, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Applications			Priority Claimed		Copy Attached	
Application Number	Country	Foreign Filing Date (MM/DD/YYYY)	YES	NO	YES	NO
9906694.6	GB	24 March 1999	Yes			No
9906696.1	GB	24 March 1999	Yes			No

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below and claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT international application(s) designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

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Parent Application Number	Filing Date	Status (Mark Appropriate Column Below)		
		Patented	Pending	Abandoned
PCT/GB00/01104	23 March 2000		Pending	

As a named inventor, I hereby revoke all prior powers and appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

KILPATRICK STOCKTON LLP, Suite 2800, 1100 Peachtree Street, Atlanta, Georgia 30309-4530

Attorney and/or Agent	Registration No.
Charles Y. Lackey	22,707
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A. Jose Cortina	29,733
James L. Ewing, IV	30,630
Charles W. Calkins	31,814
George T. Marcou	33,014
Bernard J. Graves, Jr.	33,239
Dean W. Russell	33,452
Richard T. Peterson	35,320
Charles T. Simmons	35,359
Nora M. Tocups	35,717
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Kristin L. Johnson	44,807
J. Jason Link	44,874
Bambi F. Walters	45,197
J. Michael Boggs	P46,563
Adam E. Crall	P46,646
Kyle M. Globberman	P46,730
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Cynthia B. Rothschild	P47,040

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09937068-092001

**DECLARATION FOR PATENT APPLICATION**☒ Original☐ Supplemental☐ Substitute☐ PCT

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below), or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**VACCINE COMPOSITION****(Title of the Invention)**

the specification of which (check one)

☐ is attached hereto☐ was filed on \_\_\_\_\_ as U. S. Application Serial Number \_\_\_\_\_☒ was filed as PCT International Application Number PCT/GB00/01104 on 23 March 2000  
and was amended under PCT Article 19 on 17 April 2001

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

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independent contractor and this application has been or will be assigned to such entity) and in such cases do not represent me individually. I further acknowledge I have not established, nor will I seek to establish, any personal attorney/client relationship with Kilpatrick Stockton LLP in connection with this application and understand that, should I require legal representation, I will obtain such, at my expense, other than through Kilpatrick Stockton LLP.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of first inventor Hazire Oya ALPAR  
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Inventor's signature Leslie Baillie Date 31st Aug 2001

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